



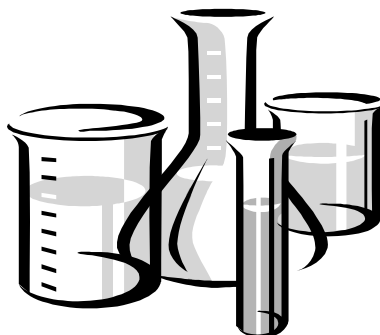
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Ecological Effects Test Guidelines

OCSP 850.4500: Algal Toxicity



NOTICE

This guideline is one of a series of test guidelines established by the United States Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug and Cosmetic (FFDCA) (21 U.S.C. 346a). Prior to April 22, 2010, OCSPP was known as the Office of Prevention, Pesticides and Toxic Substances (OPPTS). To distinguish these guidelines from guidelines issued by other organizations, the numbering convention adopted in 1994 specifically included OPPTS as part of the guideline's number. Any test guidelines developed after April 22, 2010 will use the new acronym (OCSPP) in their title.

The OCSPP harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting the test, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA, and/or the FFDCA. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. At places in this guidance, the Agency uses the word "should." In this guidance, the use of "should" with regard to an action means that the action is recommended rather than mandatory. The procedures contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the recommendations described in these guidelines, and the Agency will assess them for appropriateness on a case-by-case basis.

For additional information about these test guidelines and to access these guidelines electronically, please go to <http://www.epa.gov/ocspp> and select "Test Methods & Guidelines" on the left side navigation menu. You may also access the guidelines in <http://www.regulations.gov> grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 850.4500: Algal Toxicity.

(a) Scope—

(1) **Applicability.** This guideline is intended to be used to help develop data to submit to EPA under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a).

(2) **Background.** The source material used in developing this harmonized OCSPP test guideline includes the OPPT guideline under 40 CFR 797.1050 Algal Acute Toxicity Test; OPP 122-2 Growth and Reproduction of Aquatic Plants (Tier 1) and OPP 123-2 Growth and Reproduction of Aquatic Plants (Tier 2) (Pesticide Assessment Guidelines Subdivision J); Non-target Plants: Growth and Reproduction of Aquatic Plants Tiers 1 and 2 Standard Evaluation Procedure; OECD 201 Algal Growth Inhibition Test; ASTM E 1218-04, Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae; and OPP Pesticides Reregistration Rejection Rate Analysis: Ecological Effects. This guideline was formerly Public Draft OCSPP 850.5400 (April, 1996).

(b) **Purpose.** This guideline is intended for use in developing data on the toxicity of chemical substances and mixtures (“test chemicals” or “test substances”) subject to environmental effects test regulations. This guideline prescribes test procedures and conditions designed to determine the inhibition of growth and the pattern of growth in algae exposed to a test substance. This guideline was written specifically for the freshwater green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) and the marine diatom *Skeletonema costatum* (see paragraph (e)(3)(i) of this guideline). Use of *Navicula pelliculosa* or other test species may require some specific modifications in test procedures. Methods for toxicity testing with the cyanobacterium species *Anabaena flos-aquae* (formerly classified as a blue-green alga) can be found in OCSPP 850.4550. The Environmental Protection Agency will use data from these tests in assessing the hazard and risks a test substance may present in the aquatic environment. This guideline should be used in conjunction with OCSPP 850.4000 (Background and special considerations for conducting ecological effects tests with terrestrial and aquatic plants, cyanobacteria, and terrestrial soil core microcosms), which provides general information and overall guidance for the plant test guidelines and OCSPP 850.1000 (Background and special considerations for conducting ecological effects tests with aquatic and sediment-dwelling fauna and aquatic microcosms), which provides general information for conducting toxicity tests in an aqueous matrix.

(c) **Definitions.** The definitions in OCSPP 850.1000 and OCSPP 850.4000 are applicable to this guideline. In addition, the following more specific definitions also apply:

Algicidal refers to having the property of killing algae.

Algicidal concentration is the lowest concentration tested which allows no net growth of the population of test organisms during either exposure to the test substance or during the recovery period in the absence of test substance.

Algistatic refers to having the property of inhibiting algal growth.

Algistatic concentration is the highest concentration tested which allows no net growth of the population of test organisms during exposure to the test substance but permits regrowth when the organisms are placed in test substance-free medium.

Biomass is the dry weight of living matter present in a population and expressed in terms of a given volume, *e.g.* mg algae per liter. Because dry weight is difficult to measure accurately, surrogate measure of biomass, such as cell counts, are typically used in this test.

Growth refers to an increase in algal biomass in a specified period of time.

Static system for this test refers to a system in which old nutrient medium (test solution) is not renewed or replaced during the period of the test.

(d) General considerations—

(1) Summary of the test. Organisms of a particular species of microalgae are maintained under static conditions in test vessels containing nutrient medium alone and nutrient medium to which the test substance has been added. Over an exposure period of 96 hours, data on population growth (cell density) are obtained on a daily basis. The test is designed to determine the quantity of test substance that results in a 50 percent (50%) inhibition (IC₅₀) in biomass yield, growth rate, and the area under the growth curve. The results of the test are expressed as 96-hour median inhibition concentration (IC₅₀) values. In addition, the no observed effect concentration (NOEC) for these response measures are determined. The results are used to establish toxicity levels, evaluate hazards or risks to aquatic plants, and to indicate if further testing at a higher tier is necessary. Note historically in OCSPP pesticide and industrial chemical guidelines the term EC_x was used to cover both the current OCSPP 850.4000 definition of EC_x (concentration where *x*% of the population exhibit the effect (*e.g.*, mortality)) and IC_x (concentration resulting in a *x*% decrease or inhibition effect on an attribute of the population (*e.g.*, growth rate)).

(2) General test guidance. The general guidance in OCSPP 850.4000 and OCSPP 850.1000 applies to this guideline except as specifically noted herein.

(3) Range-finding test. A range-finding test is usually conducted to establish the appropriate test substance solution concentrations for the definitive test. In the range-finding test, the test organisms are exposed to a series of widely-spaced concentrations of the test substance (*e.g.*, 0.1, 1.0, 10, 100 milligrams per liter (mg/L), *etc.*). The details of the range-finding test do not have to be the same as the definitive testing in that there are no replicates, and the number of test organisms used, and duration of exposure may be less than in definitive testing. In addition, the types and frequency of observations made on test organisms are not as detailed or as frequently observed as that of a definitive test and results are analyzed using nominal concentrations. However, the range-finding test will be more useful the greater the similarity between the range-finding test and the definitive test.

(4) Definitive test. The goal of the definitive test is to determine the concentration-response curves, 96-hour IC₅₀ values (with 95% confidence intervals and standard error),

and NOEC and lowest observed effect concentration (LOEC) values for algal population growth based on yield, growth rate, and area under the growth curve for each species tested. The slopes of the concentration-response curves, and associated standard errors, and 95% confidence intervals of the slopes should be determined for all of the population growth measures, if possible, for each species. However, at a minimum, the full concentration-response curve (curve between IC₀₅ to IC₉₀) is determined for the most sensitive measure of effect for a given species using a minimum of five concentrations of the test chemical, plus appropriate controls. Recommend adding one or two additional test concentrations in the lower tail of the concentration-response curve for the most sensitive endpoint to ensure bracketing of the most sensitive NOEC or IC₀₅ value. For a satisfactory test for a given species, the lowest treatment concentration is below the yield, average specific growth rate, and area under the growth curve for all IC₅₀ values and is at or below the NOEC (or IC₀₅) for all growth measures. Analytical confirmation of test concentrations is performed as described in OCSPP 850.1000. A summary of test conditions is provided in Table 3 of this guideline and validity elements for an acceptable definitive test are listed in Table 4 of this guideline.

(5) **Limit test.** In some situations, it is only necessary to ascertain that both the 96-hour IC₅₀ values for growth measures are above a certain limit concentration, and that at this limit concentration there is no observable adverse effect on growth. For pesticides, a limit test has also been referred to as a Tier I test or Maximum Challenge Concentration test. In an algal limit test, at least four replicate test vessels are exposed to a single “limit concentration,” with the same number of test vessels containing the appropriate control solution(s). The multiple-concentration definitive test may be waived for a given test species if the following two conditions are met for yield, average specific growth rate and area under the growth curve. First, the “limit” treatment response is statistically less than a 50% decrease from the control response (*i.e.*, IC₅₀ value > limit concentration), and second, the limit treatment responses are not significantly reduced (or inhibited) as compared to the control response (*i.e.*, NOEC ≥ limit concentration). For most industrial chemicals, 1,000 mg/L or the limits of water solubility or dispersion are considered appropriate as the limit concentration. For pesticides, the limit concentration is equivalent to the maximum label rate (pounds of active ingredient per acre (lbs a.i./A)) directly applied to a one acre pool that is 6 inches deep (21,280 cubic feet (ft³) or 602,581 liters). For example, a 1 lb a.i./A (or 453,592 milligrams (mg) a.i. per acre) application rate and assuming a water density of 1 gram per milliliter would have a limit concentration of 0.75 mg a.i./L. Except for the number of treatment groups, an acceptable limit test follows the same test procedures, is the same duration, and has the same number of controls as the multi-concentration definitive test (Table 3). Acceptable limit tests like definitive tests include analytical confirmation of the exposure concentration.

(e) **Test standards—**

(1) **Test substance.** For industrial chemicals, the substance to be tested should be technical grade unless the test is designed to test a specific formulation, mixture, or end-use product. For pesticides, the use of the typical end-use product (TEP) instead of the technical grade active ingredient (TGAI) is preferred for all aquatic plant phytotoxicity

tests. If there is more than one TEP with the same inert substances, the one with the highest percent a.i. and/or the one most commonly used should be tested. If there is more than one TEP with different inert substances, a TEP representative of each different inert substance should be tested in the range-finding test and at a minimum the most sensitive one tested in the definitive or limit test. Adjuvants are not used with TEP or TGAI testing of algae. OCSPP 850.1000 and OCSPP 850.4000 list the type of information that should be known about the test substance before testing, and discusses methods for preparation of test substances.

(2) **Test duration.** The test duration is 96 hours.

(3) **Test organism—**

(i) **Species.**

(A) This test guideline prescribes testing conditions and standards for three algal species: the freshwater green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and also as *Raphidocelis subcapitata*); the marine diatom, *Skeletonema costatum*; and the freshwater diatom, *Navicula pelliculosa*. For pesticides all three of these species are tested at a minimum. These species have been historically used for this type of testing. For testing industrial chemicals, the specific algal species tested are selected on a case-by-case basis.

(B) Some species that may be tested in addition include *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*), *Microcystis aeruginosa*, *Thalassiosira pseudonana*, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*. Appropriate references (as given in paragraph (j)(3), (j)(7), (j)(8), (j)(9), (j)(10), and (j)(15) of this guideline) should be consulted regarding the correct culturing and testing conditions for these species.

(C) Procedures for testing the cyanobacterium, *Anabaena flos-aquae*, formerly classified as a blue-green alga, are found in OCSPP 850.4550.

(ii) **Source, age and condition.**

(A) All algae used for a particular test should be from the same source and the same stock culture. Also, the clone of all species should be specified. Algae to be used in toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Upon receipt of a culture of a species not previously maintained in the testing facility, a period of six weeks culturing is recommended to establish the ability to successfully maintain a healthy, reproducible-growing culture. The algal inoculum to begin the toxicity test should be from logarithmically-growing stock cultures (typically 3- to 7-days old).

(B) A culture should not be used for starting a test under the following conditions:

(1) If it is not in the log growth phase;

(2) If visual examination at 400 power shows it is contaminated by fungi or other algae, or if the health of the culture is doubtful in any respect;

(3) If test algae were used in a previous test, either in a treatment or a control.

(iii) Culturing procedures.

(A) Algal cultures should be maintained in freshwater or saltwater medium as described in the references listed in paragraphs (j)(1), (j)(3), (j)(6), and (j)(9) of this guideline. The cultures should be maintained under the same conditions as used for testing.

(B) Aseptic stock transfer should be performed on a regular schedule (*e.g.*, once or twice weekly) to maintain a supply of cells in or near the logarithmic growth phase. Long-term maintenance of cultures on a solid medium containing 1% agar in sterile Petri plates or test tubes may be desirable. However for a satisfactory test, the algal inoculum used to initiate toxicity testing is from a liquid culture shown to be actively growing (*i.e.* capable of logarithmic growth within the test period) in at least two subcultures lasting 7 days each prior to the start of the definitive test.

(C) Stock algal cultures of *P. subcapitata* and *N. pelliculosa* should be shaken on a rotary shaking apparatus. Culture vessels containing *S. costatum* should be shaken by hand once or twice daily. If clumping of cells is not experienced, *S. costatum* may be continuously shaken at approximately 60 cycles per minute.

(4) Administration of test substance—

(i) Preparation of test solutions—

(A) **Stock solutions or direct addition.** Test solutions are prepared by adding the test substance to the nutrient medium either as direct addition or by addition of a stock solution. Typically, a stock solution of the test substance is prepared and aliquots of the stock solution added to medium to obtain the desired test concentrations. Guidance for preparation of test solutions, especially for difficult or low solubility test substances, is provided in OCSPP 850.1000.

(B) Solvents. The recommended solvent for algal toxicity tests is N,N-dimethyl-formamide, as solvents such as acetone can cause stimulation of bacterial growth. The concentration of solvent should preferably be the same in all test treatments and should not exceed 0.1 milliliters per liter (mL/L).

(C) Stock solution pH adjustment. The pH may be adjusted in stock solutions to match the pH of the medium if pH change does not affect the stability of the test substance in the stock solution or test solution. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) may be used for this adjustment if warranted. The pH should not be adjusted after the addition of the test substance or stock solution into the test medium except as discussed in paragraph (e)(9)(iv)(C)(2) of this guideline.

(D) Exposure technique.

(1) This test is conducted using a static exposure technique. Although semi-continuous algal culturing techniques are available, they have not been commonly employed in algal toxicity testing and their use is not required.

(2) When a substance is known to have a tendency to vaporize, closed test flasks with increased head-space may be used. Attempts should be made to determine the amount of the substance which remains in solution, and extreme caution is advised when interpreting results of test with volatile chemicals using closed systems.

(ii) Treatment levels. For a given plant species a minimum of five test solution concentrations are tested for multi-concentration definitive testing, plus the appropriate control(s). A range-finding test can be used to establish the appropriate test solution concentrations for the definitive test (see paragraph (d)(3) of this guideline). For scientifically sound estimates of a given point estimate (*e.g.*, IC_{50} , IC_{05}) test substance concentrations should immediately bracket the point estimate(s) of concern. The concentrations should be a geometric progression of twofold at a minimum (*e.g.*, 0.1, 0.2, 0.4, 0.8, and 1.6 milligrams per liter (mg/L)). While a twofold progression is preferred, threefold and fourfold progressions are acceptable. If a fourfold or higher series progression is used, the rationale for using this large an interval between concentrations and the effect on the accuracy and reproducibility of the point estimate(s) and NOEC should be provided. For an acceptable study, the lowest test treatment level should be lower than the IC_{50} values for yield and average specific growth rate based on cell density. The lack of a NOEC for an effect measure is not critical as long as the response-curve for the effect measure is acceptable for calculation of the 5% inhibition concentration (IC_{05}). It is recommend that one or two additional test concentrations in the lower tail of the concentration-response curve of the most sensitive endpoint for a given species be

added to ensure bracketing of both the most sensitive IC₅₀ value and the most sensitive NOEC (or IC₀₅) value. For a limit test, there is single treatment concentration, plus the appropriate control(s). Guidance on the limit concentration is provided in paragraph (d)(5) of this guideline.

(iii) Introduction of test organisms.

(A) In preparation for the test, appropriate volumes of nutrient medium and/or test solution are placed in the test vessels. Within 30 minutes algae are then introduced into the test vessels, which are subsequently placed in a growth chamber or a laboratory testing area. Inoculum should be impartially or randomly distributed among the test vessels in such a manner that test results show no significant bias from the distributions.

(B) Test vessels within the testing area are positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement. Because illumination may vary at different positions within the growth chamber, and since this parameter has an important influence on algal growth, it is recommended that the test vessels be randomly re-positioned each day.

(5) Controls.

(i) Every test includes a negative control consisting of the same nutrient medium, conditions, procedures, and algae from the same culture, except that none of the test substance is added. In addition, vehicle (solvent) controls are also included if a solvent is used to dissolve or suspend the test substance.

(ii) For a satisfactory test, cell counts in the controls should increase by a factor of at least 100 times for *P. subcapitata* and a factor of at least 30 times for *S. costatum* by test termination (*i.e.*, logarithmic growth in the controls).

(iii) At test termination the coefficient of variation for mean control yield should be less than 15% and it should be less than 15% for average specific growth rate, which is a logarithmically-transformed variable.

(6) Inoculum concentration and replicates. The minimum number of replicates per treatment and control is four to provide acceptable confidence in the results and the ability to conduct the statistical hypothesis tests. Each test vessel should be inoculated at an initial population density to allow sufficient growth under the test conditions to provide accurate quantification without resulting in nutrient or carbon dioxide limitation. Recommended initial population density is 10,000 cells/mL for *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) and *Skeletonema costatum*. These inoculum concentrations are known to result in acceptable population densities at the end of 96 hours under the specified test parameters for these species. The use of other test species such as *Navicula pelliculosa* may require higher initial inoculum concentrations, and the initial population density necessary to result in sufficient growth over the test period should be determined on a case-by-case basis. In no case should a test be started

with less than 10,000 cells/mL. Each test vessel should contain an equal volume of test solution and the same initial inoculum volume and concentration. The volume of inoculum used should not dilute the test solution (*i.e.*, should typically be less than 2 mL). The volume of inoculum to be added to each vessel is calculated based upon the cell concentration in the stock solution, the volume in the test vessel, and the desired initial cell concentration.

(7) Facilities, apparatus and supplies. Normal laboratory equipment and especially the following are necessary:

(i) **Containers for culturing and testing algae.** Erlenmeyer flasks should be used for test vessels. The flasks may be of any volume between 125 and 500 milliliters (mL) as long as the same size is used throughout a test and the test solution volume does not exceed 50% of the flask volume. To permit gas exchange but prevent contamination, the flasks should be covered with foam plugs, stainless steel caps, Shimadzu enclosures, glass caps or screw caps. Containers and covers that may contact the test solution should be chosen to minimize sorption of test substances, and not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. The acceptability of foam plugs should be investigated prior to use because some brands have been found to be toxic. For a satisfactory test for a given test species, all test vessels and covers in the test are identical.

(ii) **Growth chamber or laboratory environment.**

(A) A growth chamber or a laboratory environment that can hold the test vessels and will maintain the air temperature, lighting intensity, and photoperiod specified in this test guideline. If necessary for the given test species, a mechanism for continuously shaking the test vessels should be incorporated into the growth chamber or controlled environment room.

(B) Facilities should be well ventilated and free of fumes that may affect the test organisms. Construction materials and equipment that may contact the stock solution, test solution, or nutrient medium should be chosen to minimize sorption of test substances and not contain substances that can be leached or dissolved into aqueous solutions in quantities that can affect the test results. Refer to OCSP 850.1000 for additional information on appropriate construction materials.

(iii) **Environmental monitoring equipment.** Equipment for determination of test conditions (*e.g.*, pH meter, photosynthetically active radiation (PAR) light sensor, *etc.*)

(iv) **Cleaning and sterilization.** Apparatus for preparing sterile nutrient media. Apparatus for sterilizing glassware and maintaining aseptic technique during culturing and testing. New test vessels may contain substances that inhibit growth of algae. They are therefore to be cleaned thoroughly and used several times to

culture algae before being used in toxicity testing. All glassware used in algal culturing or testing is to be cleaned and sterilized prior to use.

(v) **Microscope.** Microscope capable of 100 to 400 power magnification.

(vi) **Algal cell counting equipment.** An apparatus for enumerating algae, *e.g.*, hemacytometer, plankton counting chamber, or electronic particle counter. An alternative method to performing cell counts is to determine the chlorophyll *a* concentration through spectrophotometric or fluorometric methods, as described in the reference in paragraph (j)(1) and (j)(6) of this guideline.

(vii) **Nutrient media.**

(A) Water used for preparation of nutrient medium should be of reagent quality (*e.g.*, ASTM Type I water). Freshwater algal nutrient medium is prepared by adding specified amounts of reagent-grade chemicals to reagent water. Marine algal nutrient medium is prepared by adding reagent grade chemicals to synthetic salt water or filtered natural salt water, or by preparing a complete saltwater medium. Salinity for saltwater medium should be 30 parts per thousand (ppt) \pm 5 ppt.

(B) Formulation and sterilization of nutrient medium used for algal culture and preparation of test solutions should conform to those currently recommended by ASTM for freshwater and marine algal toxicity tests described in the reference in paragraph (j)(1) of this guideline and Tables 1 and 2. Chelating agents (*e.g.* ethylenediaminetetraacetic acid (EDTA)) are included in the nutrient medium for optimum cell growth. Lower concentrations of chelating agents, down to one-third of the normal concentration recommended for AAP medium, may be used in the nutrient medium used for test solution preparation if it is suspected that the chelater will interact with the test substance. Nutrient medium should be freshly prepared for algal testing or may be stored under refrigeration in the dark at 4 degrees Celsius ($^{\circ}\text{C}$). Nutrient medium should be sterilized by autoclaving or filtering (0.22 micrometer (μm) membrane filter). At the start of the test, the pH of the nutrient medium should be 7.5 ± 0.1 for freshwater algal medium and 8.0 ± 0.1 for marine algal medium. The pH may be adjusted prior to addition of the test substance with 0.1 normal (*N*) or 1*N* sodium hydroxide (NaOH) or hydrochloric acid (HCl).

(8) **Environmental conditions.** Environmental conditions during the test should be maintained as specified below:

(i) **Temperature.** The test temperature is 24 $^{\circ}\text{C}$ for *P. subcapitata* and *N. pelliculosa*, and 20 $^{\circ}\text{C}$ for *S. costatum*. Excursions from the test temperature should be no greater than ± 2 $^{\circ}\text{C}$. Temperature monitoring is described in paragraph (e)(9)(iv)(A) of this guideline.

(ii) **Lighting and photoperiod.** Test vessels containing *P. subcapitata* and *N. pelliculosa* are illuminated continuously; those containing *S. costatum* are provided a 14-hour light/10-hour dark photoperiod. Cool-white fluorescent lights providing 60 micromoles per square meter per second ($\mu\text{mol}/\text{m}^2/\text{s}$), for cool-white fluorescent lighting this is approximately equivalent to 4300 lux, are used. Additional information on the use of lighting in plant toxicity tests can be found in the references given in OCSPP 850.4000.

(iii) **Shaking.** Test vessels containing these species should be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/min during the test. The rate of oscillation should be determined at the beginning of the test or at least once daily during testing if the shaking rate is changed or changes. Test vessels containing *S. costatum* should be shaken by hand once or twice daily. If clumping of cells is not experienced, *S. costatum* may be continuously shaken at approximately 60 cycles per minute.

Table 1.—Preparation of Medium for Freshwater Algae

This medium (referred to as AAP medium) is prepared by adding 1 milliliter (mL) of each of the macronutrient stock solutions and 1 mL of the micronutrient stock solution listed below to approximately 900 mL reagent grade water and then diluting to 1 liter (L).
<p><u>Macronutrient stock solutions</u> are made by dissolving the following into 500 mL of reagent grade water:</p> <p>Sodium nitrate (NaNO_3) - 12.750 grams (g) Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) - 6.082 g Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) - 2.205 g Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) - 7.350 g Potassium phosphate (K_2HPO_4) - 0.522 g Sodium bicarbonate (NaHCO_3) - 7.500 g</p>
<p>The <u>micronutrient stock solution</u> is made by dissolving the following into 500 mL of reagent grade water:</p> <p>Boric acid (H_3BO_3) - 92.760 milligrams (mg) Manganese chloride tetrathhydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) - 207.690 mg Zinc chloride (ZnCl_2) - 1.635 mg Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) - 79.880 mg Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) - 0.714 mg Sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) - 3.630 mg Copper chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) - 0.006 mg [Typically must be prepared by serial dilution]. Ethylenediaminetetraacetic acid disodium salt dihydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) - 150.000 mg Sodium selenite pentahydrate ($\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$) - 0.005 mg [Used only in medium for stock cultures of diatom species]</p>
For freshwater diatom species only, add sodium metasilicate nonahydrate ($\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$) as another macronutrient. May be added directly (202.4 mg) or by way of stock solution to give 20 mg/L silicon (Si) final concentration in medium (see reference in paragraph (j)(1) of this guideline).
Adjust pH to 7.5 ± 0.1 with 0.1 N or 1.0 N sodium hydroxide (NaOH) or hydrochloric acid (HCl). Filter the media into a sterile container through a 0.22 micrometer (μm) membrane filter. Store medium in the dark at approximately 4 degrees Celsius ($^{\circ}\text{C}$) until use.

Table 2.—Preparation of Medium for Saltwater Algae

<p>This medium (referred to as MAA medium) is prepared by adding the aliquots of Metal Mix, Minor Salt Mix and Vitamin Mix listed below into a sterile container containing approximately 900 milliliters of natural or artificial salt water (salinity 30 ± 5 ppt) then diluting to 1 L. Either natural salt water that has been filtered through a 0.22 μm membrane filter or reconstituted salt water is used.</p> <p>Add the amounts given below to prepare medium used for toxicity testing. Add twice the amounts given to prepare medium for use in maintenance of stock cultures.</p> <p>Add 15 mL of Metal Mix</p> <p>Add 10 mL of Minor Salt Mix stock solution</p> <p>Add 0.5 mL of Vitamin Mix stock solution (Add 1 mL of vitamin mix if <i>Thalassiosira</i> is used).</p>
<p>The Metal Mix is prepared by adding the specified amount of chemicals in the order listed below to 900 milliliters (mL) reagent water and diluting to 1 liter (L).</p> <p><u>Metal Mix:</u></p> <p>Ferric chloride hydrate ($\text{FeCl}_3 \cdot \text{H}_2\text{O}$) - 0.048 g</p> <p>Manganese chloride tetrathhydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) - 0.144 g</p> <p>Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) - 0.045 g</p> <p>Copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) - 0.157 mg</p> <p>Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) - 0.404 mg</p> <p>Boric acid (H_3BO_3)- 1.140 g</p> <p>Ethylenediaminetetraacetic acid disodium salt dihydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) - 1.0 g</p>
<p>The Minor Salt Mix is prepared by adding the specified amounts of the chemicals listed below to 900 mL reagent water and diluting to 1 L.</p> <p><u>Minor Salt Mix:</u></p> <p>Potassium phosphate (K_3PO_4) - 0.3 g</p> <p>Sodium nitrate (NaNO_3) - 5.0 g</p> <p>Sodium metasilicate nonahydrate ($\text{NaSiO}_3 \cdot 9\text{H}_2\text{O}$) - 2.0 g</p>
<p>The Vitamin Mix is prepared by adding the specified amount of chemicals in the order listed below to 900 mL reagent water and diluting to 1 L.</p> <p><u>Vitamin Mix:</u></p> <p>Thiamine hydrochloride - 500 mg</p> <p>Biotin - 1 mg</p> <p>cyanocobalamin (vitamin B_{12}) - 1.0 mg</p>
<p>Adjust MAA medium pH to 8.0 ± 0.1 with 0.1 N or 1.0 N NaOH or HCl. Store medium in the dark at approximately 4 °C until use.</p>

(9) Observations—

(i) **Measurement of test substance.** Analytical confirmation of dissolved test concentrations is performed at a minimum at test initiation and at test termination for static tests, as described in OCSP 850.1000. The analytical methods used to measure the amount of test substance in a sample are validated before beginning the test, as described in OCSP 850.1000. Samples for analysis should be collected as described in OCSP 850.1000, with the following exception: at the end of the test and after aliquots have been removed for algal growth-response determinations, microscopic examination, mortal staining, or subculturing, the replicate test containers for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the dissolved concentration of test substance in solution determined after all algal cells have been removed, either by centrifugation or filtration. The effect of centrifugation or filtration upon recovery of the test substance should be determined during method validation. As an additional procedure, the mass of test substance sorbed

to the algae may be determined. To measure sorbed test substance, separate and concentrate the algal cells from the remaining pooled sample by centrifuging or filtering and measure the test substance concentration in the algal-cell concentrate.

(ii) **Test solution appearance.** Observations are made daily on test substance solubility (*e.g.*, surface slicks, clarity, precipitates, or material adhering to the sides of the test vessels) and recorded.

(iii) **Dilution water quality.** The dilution water source used to prepare media should be periodically analyzed to document and characterize the hardness, alkalinity, pH, conductivity, total organic carbon (TOC) or chemical oxygen demand magnitude and variability, and to ensure that pesticides, PCBs and toxic metals are not present at concentrations that are considered toxic. See OCSP 850.1000 for guidance on dilution water.

(iv) **Environmental conditions—**

(A) **Temperature.** It is impractical to measure the temperature of the solutions in the test vessels while maintaining axenic conditions. Therefore, one or two extra test vessels may be prepared for the purpose of measuring the solution temperature during the test. Alternatively, hourly measurements of the air temperature (or daily measurements of the maximum and minimum) are acceptable. Because the test vessels are placed in an environmental chamber or incubator, the air temperature is more likely to fluctuate than the water temperature.

(B) **Light intensity.** Light intensity should be monitored at test initiation at the approximate level of the test solution at each test vessel position in the growth chamber or environmental control room. If it is suspected during the test that light intensity has changed by 15% or more, monitoring of light intensity should be conducted daily. A photosynthetically active radiation (PAR) sensor is used to measure light quality. The light intensity should not vary more than $\pm 15\%$ from the selected light intensity at any test vessel position in the incubator or growth chamber. Because illumination may vary at different positions, and since this environmental parameter has an important influence on growth, it is recommended that the test vessels be randomly re-positioned on a regular schedule (*e.g.*, daily) to minimize spatial differences.

(C) **pH.**

(1) The pH of all test solutions is measured at test initiation and termination. This measurement may be made on the bulk test solutions at test initiation and on samples of pooled replicates of each test treatment at test termination provided none of the

replicates appear to be “outliers” with respect to growth, in which case individual pH measurements should be made.

(2) Before addition of the inoculum, if the test substance is highly acidic and reduces the pH of the test solution below 5.0 at the first measurement, or is highly basic and increases the pH of the test solution similarly, appropriate adjustments to pH should be considered and the test solution measured for pH on each day of the test.

(v) Measures of effect—

(A) Cell count. At the end of 24, 48, 72, and 96 hours, at a minimum the algal cell density (cells per milliliter) in all test vessels are enumerated by performing cell counts using direct microscopic observation or using an electronic particle counter. Additional confirmation measurements include chlorophyll *a* (measured spectrophotometrically or fluorometrically).

(1) Microscopic counting. Microscopic counting of cells can be performed using a hemacytometer or an inverted microscope with settling chambers. Precision is proportional to the square root of the number of cells counted. For microscopic counting, two samples should be taken from each test vessel and two counts made of each sample. Whenever feasible, at least 400 cells per test vessel should be counted in order to obtain $\pm 10\%$ accuracy at the 95% confidence level.

(2) Electronic particle counter. An alternative method to enumerate large numbers of cells very rapidly is to use an electronic particle counter. To use this method, an electronic particle count for a sample is converted to a cell count using data developed by the laboratory demonstrating the correlation between electronic particle counts and microscopic counts for each algal test species. Automated particle counting, although the most rapid and sensitive method, has limitations, some related to particle interferences. If the test solution does not have a low background in the particle size range of the test species, masking errors will result. An additional test vessel at each concentration containing test substance and growth medium but no algae can allow measurement of, and if needed, correction for, potential particle interference.

(B) Appearance and condition. Note daily any unusual cell shapes, color differences, differences in chloroplast morphology, flocculations, adherence of algae to test vessels, or aggregation of algal cells. These observations are qualitative and descriptive, and are not used in endpoint

calculations. They can be useful in determining additional effects of test substances. In addition, microscopic observations at test termination are performed to determine whether the altered growth response between controls and test algae (at the concentrations of test substance demonstrating an effect) was due to a change in relative cell numbers, cell sizes, or both.

(C) Algistatic and algicidal determination. At the end of the definitive test, determination of algistatic and algicidal effects may be performed. When performed algistatic effects may be differentiated from algicidal effects by either Method 1 or Method 2 described in paragraphs (e)(9)(v)(C)(1) and (e)(9)(v)(C)(2) of this guideline, respectively.

(1) Method 1. Add 0.5 mL of a 0.1% solution (weight/volume) of Evans blue stain to a 1-mL aliquot of algal suspension from a control vessel and to a 1-mL aliquot of algae from the test vessel having the lowest concentration of test substance which completely inhibited algal growth. If algal growth was not completely inhibited, select an aliquot of algae for staining from the test vessel having the highest concentration of test substance that inhibited algal growth. Wait 10 to 30 minutes, examine microscopically, and determine the percent of the cells that stain blue (indicating cell mortality). A staining control is also performed concurrently using heat-killed or formaldehyde-preserved algal cells; 100% of these cells should stain blue. This method will work for *S. costatum* and possibly *Navicula* spp., but probably will not work with *P. subcapitata*.

(2) Method 2. Remove 0.5 mL aliquots of test solution containing growth-inhibited algae from each replicate test vessel having the lowest concentration of test substance that completely inhibited algal growth. If algal growth was not completely inhibited, select aliquots from the highest concentration of test substance that inhibited algal growth. Combine these aliquots into a new test vessel and add a sufficient volume of fresh nutrient medium to dilute the test substance to a concentration that does not affect growth (using the original test vessel size and solution volume is generally appropriate). Aliquots from the control test vessels are also transferred to clean medium. Incubate these subcultures under the environmental conditions used in the definitive test for a period of up to 9 days, and observe periodically (e.g., every other day) for algal growth (direct or indirect methods) to determine if the algistatic effect noted after the 96-hour definitive exposure test is reversible. This subculture test may be discontinued as soon as growth occurs.

(f) Treatment of results—

(1) Response variable calculation. Algal population density is the biomass measurement normally used in this test guideline to evaluate the inhibitory and stimulatory effects of the test substance. Three response variables are calculated from algal density: final population density, also referred to as yield, as described in paragraph (f)(1)(i) of this guideline; average specific growth rate, as described in paragraph (f)(1)(ii) as described in this guideline; and area under the growth curve, as described in paragraph (f)(1)(iii) in this guideline.

(i) Final population density or yield. To correctly represent yield, the initial biomass values should be subtracted from the final biomass values for each test vessel. Since the initial values are extremely small relative to the final values, this correction has a small impact upon the test results but is nonetheless recommended.

$$Y = b_1 - b_0 \quad \text{Equation 1}$$

where:

Y = final yield of observed biomass (cell density)

b_0 = biomass (cell density) at test initiation

b_1 = biomass (cell density) at test termination

(ii) Average specific growth rate. Average specific growth rate (sometimes called relative growth rate) is the rate of growth over a given time interval. The growth rate cell density for each test vessel (replicate) over a given time interval is calculated as given in Equation 2. At a minimum, the average specific growth rate is calculated for the time interval between test initiation and termination. The average specific growth rate during the course of the test (days 0 to 1, 1 to 2, 2 to 3, *etc.*), also called the section-by-section growth rate, is calculated also for each test vessel to assess effects on the pattern of growth of the test substance occurring during the exposure period, such as an increased lag phase.

$$\bar{r}_{i-j} = \frac{\ln(b_j) - \ln(b_i)}{t} \quad \text{Equation 2}$$

where:

\bar{r}_{i-j} = average specific growth rate per day (day^{-1}) of observed biomass (cell density) from time i to j .

b_i = observed biomass (cell density) at beginning of the observation interval, time i

b_j = observed biomass (cell density) at end of the observation interval,
time j

t = time interval from i to j in days

(iii) **Area under the growth curve.** The area under the growth curve for each test vessel (replicate) based on cell density is calculated using Equation 3. At a minimum the area under the growth curve from test initiation through termination is calculated.

$$A = \left(\frac{b_1 - b_0}{2} \right) (t_1) + \left(\frac{b_1 + b_2 - 2b_0}{2} \right) (t_2 - t_1) + \left(\frac{b_{n-1} + b_n - 2b_0}{2} \right) (t_n - t_{n-1}) \quad \text{Equation 3}$$

where:

A = area under the growth curve for biomass (cell density)

b_0 = observed biomass (cell density) at test initiation

b_1 = observed biomass (cell density) at time t_1

b_2 = observed biomass (cell density) at time t_2

b_n = observed biomass (cell density) at time t_n

t_1 = time (units day) of the first measurement after test initiation

t_n = time (day) of the n^{th} measurement after test initiation

(2) Summary statistics—

(i) **Environmental conditions.** Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) by treatment level for temperature and pH. Calculate descriptive statistics (mean, standard deviation, coefficient of variation, minimum, maximum) by test vessel position for light intensity.

(ii) **Test substance concentration.** Calculate descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation) by test vessel and treatment level of the test substance soluble concentration. For each treatment level, compare the initial test substance concentration with test substance concentration at the end of the test in each treatment. If the test substance was not stable calculate a rate of decline of the test substance; a time-weighted mean concentration should be calculated under these circumstances (*e.g.*, exponential decay calculate the area under the exponential decay concentration curve divided by the total exposure days). For pesticides under unstable test substance conditions, the measured test substance concentration at test initiation is considered appropriate rather than the time-weighted average

because of the exposure estimate currently used by OPP for calculating risk estimates.

(iii) **Cell density.** For each treatment level and observation time calculate the mean, standard deviation, and coefficient of variation for algal cell density. Calculate the mean treatment yield, average specific growth rate, and area under the growth curve based on changes in algal cell density from test initiation to test termination. Calculate the mean treatment section-to-section growth rate for cell density.

(iv) **Appearance and condition.** Morphological symptoms of plant injury should be summarized in tabular form by time of observation, treatment, and replicate. Definition of any index values used for morphological symptoms, indicating the severity of the symptom(s), should be provided.

(3) Percent inhibition—

(i) **Treatment level.** For yield, average specific growth rate, and area under the growth curve calculate the percent inhibition (%I) at each treatment level at 96 hours using Equation 4.

$$\%I = \frac{(C - X)(100)}{C} \quad \text{Equation 4}$$

where:

C = the control mean response value (yield, average specific growth rate, and area under the growth curve); and

X = the treatment mean response value (yield, average specific growth rate, and area under the growth curve, respectively). Stimulation is reported as negative %I.

(ii) **Growth pattern.** If there are substantial differences between the section-by-section growth rates and the average growth rates for a test vessel this indicates deviation from theoretical exponential growth. In this instance, compare specific growth rates from exposed cultures during the time period of maximum inhibition to those for controls during the same period. The same time interval should be used for each test vessel in all treatments.

(4) **Doubling time of controls.** The doubling time (T_d) of cell density in controls at test termination is calculated using Equation 5.

$$T_d = \ln(2) / \bar{r}_{Control} \quad \text{Equation 5}$$

where:

$\bar{r}_{Control}$ = mean of the control average specific growth rate test vessel values from paragraph (f)(1)(ii) of this guideline.

(5) **Limit test—**

(i) **IC₅₀ values.** To ascertain that the yield, average specific growth rate, and area under the growth curve 96-hour IC₅₀ values based on algal cell density occur above the “limit” concentration, a one-sided test which compares the difference between two sample groups to a fixed value (or difference) is performed for each of these response measures. For a comparison of sample means, the difference defining the IC₅₀ compared to controls is operationally defined as a 50% reduction or inhibition from the control sample mean (Equation 6). The null hypothesis (H_0) stated in terms of true population parameters is that the difference of the “limit” treatment mean response (μ_{limit}) from the control mean response ($\mu_{control}$) is greater than or equal to a 50% reduction, compared to the control (*i.e.*, $H_0: \mu_{control} - \mu_{limit} \geq \delta_0$). The alternative hypothesis (H_A) is that this difference is less than a 50% reduction, compared to the control ($H_A: \mu_{control} - \mu_{limit} < \delta_0$). An example of a parametric two-sample comparison test is the Student’s t-test. If the null hypothesis is rejected, the inhibition level for the given response measure (*i.e.* yield or the average specific growth rate based on cell density) in the limit treatment as compared to the control is declared to be less than 50% (*i.e.*, IC₅₀ > limit concentration). If the null hypothesis is not rejected, the limit treatment as compared to the control response is declared to be 50% or greater (*i.e.*, IC₅₀ < limit concentration).

$$\delta_0 = (\bar{x}_{control}) \times (p/100) \quad \text{Equation 6}$$

where:

δ_0 = difference between two parameters, defined in this case as a $p\%$ reduction from the control sample mean;

$\bar{x}_{control}$ = control sample mean response (*e.g.*, yield or the average specific growth rate based on cell density); and

p = percent reduction from the control sample mean, which is 50 in the case of the IC₅₀.

(ii) **NOEC.** To ascertain that there is no observable effect at the limit treatment (*i.e.*, $\text{NOEC} \geq \text{limit concentration}$) for a given response measure (yield or the average specific growth rate based on cell density), the limit treatment response is compared to the control treatment response using a one-sided two-sample parametric or nonparametric test, as appropriate (see OCSPP 850.4000). The minimum significant difference detectable by the test or a similar estimate of the sensitivity of the test should be determined and reported.

(iii) **Multiple-dose definitive testing.**

(A) A multiple-dose definitive test is performed for a given test species if either the effect or inhibition level for one or more response measures (*i.e.*, yield or the average specific growth rate based on cell density) in the limit treatment as compared to the control response at test termination are declared to be 50% or greater effect (*i.e.*, the null hypothesis is not rejected) or the NOEC is less than the limit concentration.

(B) Multiple-dose definitive testing may be waived for a given test species if at test termination the “limit” treatment response is both statistically less than a 50% decrease from the control response and there is no observable adverse effect from the control response for all measures of effect (yield or the average specific growth rate based on cell density).

(6) **Multiple-dose definitive test—**

(i) **Concentration-response curve, slope and IC_{50} .** For concentration-response tests statistical procedures are employed to calculate the 96-hour IC_{50} value (standard error and 95% confidence interval) for yield, average specific growth rate, and area under the growth curve based on cell density. If a concentration-response curve model was fit to the data to determine an IC_{50} value, the model parameters (*e.g.*, slope) and their uncertainty estimates (*e.g.*, standard error) should be recorded. The values for each test vessel, not the mean for each treatment, should be used as the response variable in fitting the model.

(ii) **NOEC.** The 96-hour NOEC values for yield, average specific growth rate, and area under the growth curve based on cell density are determined. For pesticides if a 96-hour NOEC value can not be determined the 96-hour IC_{05} value (standard error and 95% confidence interval) is estimated and used in place of the NOEC. For industrial chemicals, the specific IC_x used in place of a NOEC that can not be determined will vary, consult with the Agency. Methods, assumptions, and results of the statistical approaches used should be recorded.

(iii) **Statistical methods.** Statistical procedures for modeling continuous toxicity data are available and should be used (see references in paragraphs (j)(2), (j)(4) and (j)(14) of this guideline and OCSPP 850.1000). Additional discussion about endpoints and statistical procedures is found in OCSPP 850.1000 and OCSPP 850.4000.

(iv) **Algicidal and Algistatic concentrations.** If applicable, results from the staining procedure or the recovery phase are used to determine the algistatic concentration (refer to paragraph (e)(9)(v)(C) of this guideline).

(g) **Tabular summary of test conditions.** Table 3 lists important conditions that should prevail during the definitive test. Except for the number of test concentrations, Table 3 also lists the important conditions that should prevail during a limit test. Meeting these test conditions will greatly increase the likelihood that the completed test will be acceptable or valid.

Table 3.—Summary of Test Conditions for the Algal Toxicity Test

Test type	Static
Test duration	96 hours
Test matrix	Synthetic growth medium appropriate for the test species
Temperature	24 °C for <i>P. subcapitata</i> and <i>N. pelliculosa</i> ; 20 °C for <i>S. costatum</i> . Constant during test within ± 2 °C.
Light quality	Cool-white fluorescent
Light intensity	60 $\mu\text{mol}/\text{m}^2/\text{s}$
Photoperiod	Continuous light for <i>P. subcapitata</i> and <i>N. pelliculosa</i> ; 14 hour light:10 hour dark for <i>S. costatum</i>
Shaking	Continuous at 100 oscillations per minute for <i>P. subcapitata</i> and <i>N. pelliculosa</i> ; manual, once or twice daily, for <i>S. costatum</i>
Salinity	30 \pm 5 ppt for saltwater species (<i>S. costatum</i>)
Test vessel size	125 - 500 mL Erlenmeyer flasks
Test solution volume	Less than or equal to 50% of the volume of the test vessel
Age of inoculum	From logarithmically-growing stock cultures (typically 3 - 7 days old)
Inoculum concentration	10,000 cells per milliliter (cells/mL) for <i>P. subcapitata</i> and <i>S. costatum</i> . At least 10,000 cells/mL for other species. Inoculum volume less than 2 mL.
Number of replicate test vessels per concentration	Four (minimum)
Test concentrations	Unless performing limit test, minimum of 5 test concentrations plus appropriate controls
Test concentration preparation	Aqueous solutions prepared by adding test substance to synthetic nutrient medium, directly or via vehicle
Measures of effect (Measurement endpoints)	96-hour IC_{50} and NOEC (or IC_{05}) values for yield, average specific growth rate, and area under the growth curve based on algal cell density

(h) **Test validity elements.** This test would be considered to be unacceptable or invalid if one or more of the conditions in Table 4 occurred or one or more performance objectives in Table 4 were not met. This list should not be misconstrued as limiting the reason(s) that a test could be found unacceptable or invalid. However, except for the conditions listed in Table 4 and in OCSPP 850.4000, it is unlikely a study will be rejected when there are slight variations from guideline environmental conditions and study design unless the control organisms are significantly affected, the precision of the test is reduced, the power of a test to detect differences is reduced, and/or significant biases are introduced in defining the magnitude of effect on measurement endpoints as compared to guideline conditions. Before departing significantly

from this guideline, the investigator should contact the Agency to discuss the reason for the departure and the effect the change(s) will have on test acceptability. In the test report, all departures from the guideline should be identified, reasons for these changes given, and any resulting effects on test endpoints noted and discussed.

Table 4.—Test Validity Elements for the Algal Toxicity Test

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1. All test vessels and closures were not identical.
 2. Treatments were not randomly assigned to test vessels, and test vessels were not randomly assigned to positions in the growth chamber.
 3. A medium (untreated) control [and solvent (vehicle) control, when a solvent was used] was not included in the test.
 4. The concentration of solvent in the range used affected growth of the test species.
 5. During the 96 hour test period, cell counts in the controls did not increase by a factor of at least 100 times for *P. subcapitata* and a factor of at least 30 times for *S. costatum* (*i.e.*, logarithmic growth in the controls was not reached during the test).
 6. A minimum of five test concentrations were not used in the definitive test.
 7. Controls were contaminated with the test substance.
 8. The lowest test concentration level was not less than the 96-hour yield, average specific growth rate, and area under the growth curve IC_{50} values based on cell density.
 9. For testing with industrial chemicals a surfactant or dispersant was used in the preparation of a stock or test solution.
 10. Temperature and light intensity were not measured as specified during the test.
-

(i) Reporting—

(1) Background information. Background information to be supplied in the report consists at a minimum of those background information items listed in paragraph (j)(1) of OCSPP 850.4000.

(2) Guideline deviations. Provide a statement of the guideline or protocol followed. Include a description of any deviations from the test guideline or any occurrences which may have influenced the results of the test, the reasons for these changes, and any resulting effects on test endpoints noted and discussed.

(3) Test substance.

(i) Identification of the test substance: common name, IUPAC and CAS names, CAS number, structural formula, source, lot or batch number, chemical state or form of the test substance, and its purity (*i.e.* for pesticides, the identity and concentration of active ingredient(s)).

- (ii) Storage conditions of the test chemical or test substance and stability of the test chemical or test substance under storage conditions if stored prior to use.
- (iii) Methods of preparation of the test substance, stock solutions, and the treatment concentrations used in the range-finding and definitive test, or limit test.
- (iv) If a vehicle (solvent) is used to prepare stock or test substance provide: the name and source of the vehicle, the nominal concentration(s) of the test substance in the vehicle in stock solutions or mixtures, and the vehicle concentration(s) used in the treatments and solvent control.

(4) Plant test species.

- (i) Scientific and common name, plant family, and strain.
- (ii) Source and method of species and strain verification.
- (iii) Culture practices, including culturing media used, and conditions.
- (iv) Acclimation period, if applicable.
- (v) Age (stage) of inoculum at test initiation.

(5) Test system and conditions. Description of the test system and conditions used in the definitive or limit test, and any preliminary range-finding tests.

- (i) Description of the growth chamber, or laboratory location, type of lights, and oscillation rates and type of apparatus.
- (ii) Description of the test vessels: size, type, material, fill volume.
- (iii) Volume of test solution in the test vessels.
- (iv) Description of preparation of inoculum used to begin test.
- (v) Inoculum volume and density added to each test vessel.
- (vi) Number of test vessels (replicates) per treatment level and control(s).
- (vii) Description of the preparation of the synthetic growth media used including the preparation date, concentration of all constituents, the initial pH, and storage conditions and duration prior to use in test.
- (viii) Description of the dilution water and any water pretreatment: source/type; pH; total organic carbon content; particulate matter content; metals, pesticides, and chlorine concentration. Describe the frequency and sample date(s) for documenting dilution water quality.

- (ix) Methods used for treatment randomization and assignment of inoculum to test vessels.
- (x) Date of introduction of test organisms to test solutions and test duration.
- (xi) Exposure technique.
- (xii) The photoperiod and light source.
- (xiii) Methods and frequency of environmental monitoring performed during the definitive or limit study for temperature, light intensity, pH.
- (xiv) Methods and frequency of measuring test substance to confirm exposure concentrations.
- (xv) Methods and frequency of measuring cell density, and any other measurements or observations of phytotoxic symptoms.
- (xvi) For the definitive and limit test, all analytical procedures should be described. The accuracy of the method, method detection limit, and limit of quantification should be given.

(6) Results.

- (i) Tabulation of test substance analytical results by test vessel and treatment (provide raw data) and descriptive statistics (mean, standard deviation, minimum, maximum, coefficient of variation).
- (ii) Environmental monitoring data results (test solution or air temperature, light intensity, and pH) in tabular form (provide raw data for measurements not made on a continuous basis), and descriptive statistics (mean, standard deviation, minimum, maximum).
- (iii) For preliminary range-finding tests, if conducted, the cell density at each treatment level and in the control(s). A description and count of morphological phytotoxic effects, if recorded, at each treatment level and in the control(s).
- (iv) For a limit test, tabulate for the limit concentration and the control(s) by replicate, the cell density at test initiation, at 24, 48, and 72 hours, and at test termination, the treatment means and standard deviations (provide the raw data).
- (v) For the definitive test, tabulation by test vessel and treatment of cell density at test initiation, at 24, 48, and 72 hours, and at test termination and treatment means and standard deviations (provide the raw data).
- (vi) For the limit and definitive tests, tabulation by test vessel and treatment of yield, average specific growth rate, and area under the growth curve for cell density.

(vii) For the limit and definitive tests, tabulation of the mean treatment yield, average specific growth rate, and area under the growth curve values, treatment standard deviations for these variables, and the treatment %I (or stimulation) in yield, average specific growth rate, and area under the growth curve relative to the control values.

(viii) For the limit and definitive test, tabulation of observed morphologic signs of toxicity (size, abnormal shape or color changes, and any other observed effect) by observation time, treatment level and replicate.

(ix) Graphs of the 96-hour concentration-response data for yield, average specific growth rate, and area under the growth curve based on cell density.

(x) For a limit test, provide the results of hypothesis tests.

(xi) For the limit test, provide a description of the statistical methods used including software package, and the basis for the choice of method.

(xii) For the definitive study and for those effect measures (yield, average specific growth rate, and area under the growth curve for cell density) with data sufficient to fit a concentration-response relationship, tabulation of the slope of the concentration-response curve and its standard error and 95% confidence limits and any goodness of fit results.

(xiii) For the definitive test, tabulation of 96-hour IC_{50} values for yield, average specific growth rate, and area under the growth curve for cell density.

(xiv) For the definitive test, a tabulation of the 96-hour NOEC and LOEC values for each response variable (yield, average specific growth rate, and area under the growth curve based on cell density). For pesticides, the IC_{05} and 95% confidence interval should be reported for response data where an NOEC could not be determined.

(xv) Description of statistical method(s) used for point estimates, including software package, for determining IC_{50} values, fitting the dose-response model, and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(xvi) Description of statistical method(s) used for NOEC and LOEC determination, including software package, and the basis for the choice of method. If an IC_{05} value is used in place of a NOEC provide a description of statistical method(s) used for point estimates, including software package, for determining IC_{05} values, fitting the dose-response model, and the basis for the choice of method. Provide results of any goodness-of-fit tests.

(xvii) If determined, report the algistatic and algicidal concentrations.

(j) **References.** The following references should be consulted for additional background material on this test guideline.

- (1) American Society for Testing and Materials. ASTM E 1218-04. Standard guide for conducting 96-h toxicity tests with microalgae. In: Annual Book of ASTM Standards, Vol. 11.06, West Conshohocken, PA. Current edition approved 2004.
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